DETERMINING THE ROLE OF DNTTIP1

Student Author

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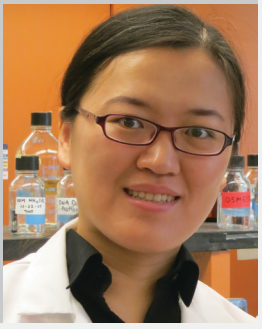
Mentors

Chris Bidwell is a professor in the Department of Animal Sciences. His areas of expertise are genetics and molecular biology. Bidwell obtained his BS and MS at Purdue University and his PhD at the University of California, Davis. He has conducted research studying genes that regulate growth and reproduction in birds, mice, fish, and several livestock species. Bidwell currently is using functional genomics and cell biology to study muscle growth in callipyge sheep.



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Abstract

Callipyge sheep have muscle hypertrophy in their loin and pelvic limbs due to a mutation in the *DLK1-DIO3* imprinted gene cluster, which results in a 30–40% increase in muscle mass without altering live weight. There is also a change in the myosin gene expression causing an increase in fast twitch glycolytic fibers. Previous gene expression studies have shown that *DNTTIP1* (terminal deoxynucleotidyl transferase interacting protein 1) is up-regulated in the callipyge muscle. *DNTTIP1* is a transcription factor, meaning it binds to a specific DNA sequence to control the amount of mRNA produced. *DNTTIP1* may regulate other genes, ultimately leading to muscle hypertrophy. The objectives of this project were to (1) confirm the cellular localization of *DNTTIP1* and (2) determine if *DLK1* and *DNTTIP1* expression can alter myosin promoter activity. Immunohistological detection of *DNTTIP1* in transfected C2C12 cells confirmed its nuclear localization. The effect of *DLK1* and *DNTTIP1* on myosin promoter activity in mouse primary myotubes was tested using luciferase reporter assays. Overexpression of *DLK1* and *DNTTIP1* were shown to significantly increase *MYH4* (fast twitch glycolytic) promoter activity and decrease *MYH7* (slow twitch oxidative) promoter activity. These results were consistent with changes in myosin gene expression in callipyge muscle, indicating that *DNTTIP1* has a role in regulating callipyge-induced muscle hypertrophy.

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Keywords

animal sciences, genetics, sheep, agriculture, callipyge, polar overdominance, muscle hypertrophy, *DNTTIP1*, gene expression, effector gene reporter assay

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DETERMINING THE ROLE OF DNTTIP1:

Piecing Together the Callipyge Sheep Muscle Hypertrophy Pathway

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INTRODUCTION

The world population is expected to exceed 9 billion people by 2050. The Food and Agriculture Organization of the United Nations projects that there will be a 73% increase in meat demand by 2050, and this is unlikely to be met by increasing animal numbers (FAO, 2011). Therefore, there is a need for substantial increases in the efficiency of meat production in livestock. Callipyge sheep display postnatal muscle hypertrophy, most prominently in the loin and pelvic limbs (see Figure 1). As depicted in Figure 2, the muscle hypertrophy results in 30-40% increased muscle mass without altering live weight, decreased fat deposition, and increased feed efficiency (Jackson, Miller, & Green, 1997; Freking, Keele, Nielsen, & Leymaster, 1998). Feed efficiency is the ratio of the pounds of feed consumed to the pounds of weight gain, and improving feed efficiency means reducing that ratio. Callipyge sheep show that substantial increases in meat production with improved feed efficiency are biologically possible.

The word callipyge is derived from the Greek "*calli,*" meaning beautiful, and "*pyge,*" meaning buttocks. The callipyge phenotype first appeared in 1983 in a flock of Dorset sheep. The affected ram, named Solid Gold, subsequently passed on the mutation to his offspring (Cockett et al., 1996). Matings between Solid Gold and normal ewes demonstrated that 50% of the offspring with the callipyge mutation were male and 50% were female, indicating segregation of the trait does not depend on sex; there is an equal chance the mutation could occur in both sexes (Georges, Charlier, & Cockett, 2003). The loin and pelvic limb hypertrophy begins at 4–6 weeks of age

Figure 1. Increased muscle mass in callipyge lambs. The development of muscle hypertrophy in the loin and hindquarters occurs during early postnatal growth and is apparent by 30 days of age. Lambs without the callipyge mutation don't display the characteristic muscle hypertrophy. The callipyge lamb is shown on the right, and the normal lamb is shown on the left. Courtesy of Chris Bidwell.

due to the development of larger type II glycolytic fibers as well as the presence of more type II glycolytic fibers (Carpenter, Rice, Cockett, & Snowder, 1996; Jackson et al., 1997; Duckett, Snowder, & Cockett, 2000).

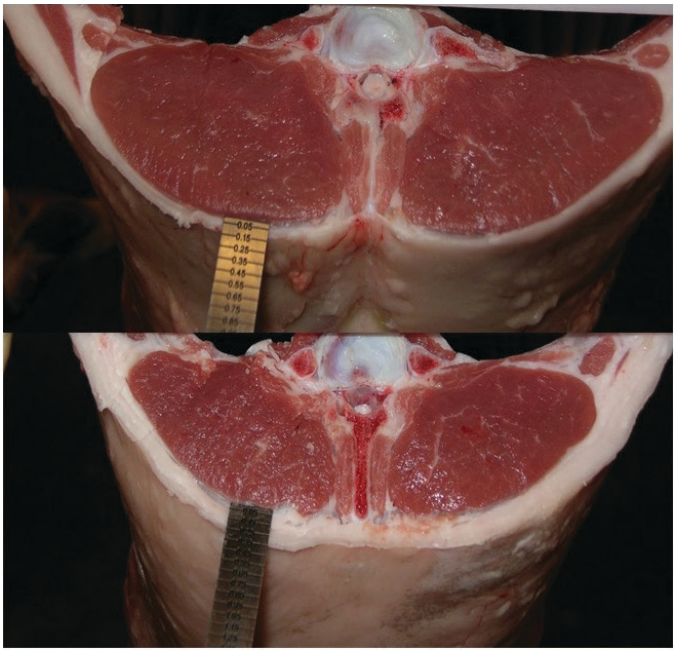

Figure 2. Rib cuts in callipyge lambs**.** Muscle hypertrophy of the loin and decreased fat in callipyge lambs are shown in two lamb carcasses at 150 days old. The callipyge lamb rib cut is shown on the top, and the normal lamb rib cut is shown on the bottom. Courtesy of Chris Bidwell.

Prior studies have been conducted to determine the method of inheritance for the callipyge gene. The callipyge gene is inherited in a non-Mendelian fashion called polar overdominance (Cockett et al., 1996). Lambs must have inherited the CLPG mutation from the sire and a normal allele from the dam to develop muscle hypertrophy. Lambs that inherit the CLPG allele from both the sire and the dam, or who inherit the CLPG allele from the dam and a normal allele from the sire, do not undergo muscle hypertrophy (Cockett et al., 1996).

Callipyge muscle hypertrophy is caused by a single nucleotide polymorphism in the *DLK1-DIO3* imprinted gene cluster on ovine chromosome 18. This mutation causes an elevated expression of the two paternal allele-specific genes, *Delta-like 1* (*DLK1*) and *Retrotransposon-like 1* (*RTL1*; Charlier et al., 2001). The hypertrophy condition results from changes in myosin gene expression, including an increase in fast twitch glycolytic fibers (*MYH4*) and a decrease in slow twitch oxidative fibers (*MYH7*; Carpenter et al., 1996). To determine the biochemical pathways of the callipyge phenotype, microarray studies of gene expression in callipyge muscle were completed. These gene expression studies have shown that *DNTTIP1* (terminal deoxynucleotidyl transferase interacting protein 1) is up-regulated in the callipyge muscle and is part of the biochemical pathway triggered by the elevated expression of *DLK1* (Fleming-Waddell et al., 2007).

DNTTIP1 was first identified by screening a human thymus cDNA library (Kubota, Maezawa, Koiwai, Hayano, & Koiwai, 2007). *DNTTIP1* appears to be a 37-kDa transcriptional cofactor that contains three DNA binding domains, including a helix-turn-helix motif and two AT-hook (ALM1 and ALM2) motifs (Kubota et al., 2007). The helix-turn-helix motif in the structure of *DNTTIP1* is responsible for binding the major grooves of DNA. The AT-hook motifs create loops that are capable of binding to AT-rich DNA sequences such as those in matrix attachment regions. Matrix attachment regions are involved in biological processes such as replication, transcription, demethylation, and chromatin accessibility (Kubota et al., 2007). *DNTTIP1* also up-regulates terminal deoxynucleotidyl transferase (TdT), which can act as a DNA polymerase (Yamashita et al., 2001). Several myosin genes contain AT-rich sequences in their promoters, which suggest that *DNTTIP1* would bind to myosin promoter sequences.

The hypothesis for this experiment is that *DNTTIP1* plays a direct role in regulating myosin gene expression. To test the hypothesis, the following objectives were developed: (1) to determine the cellular localization of *DNTTIP1* and (2) to determine if *DLK1* and *DNTTIP1* expression can alter myosin promoter activity using a luciferase reporter assay in cultured primary mouse myotubes. Identifying the biochemical and physiological pathways that control callipyge-induced muscle hypertrophy will enable more accurate selection of livestock for improved muscle growth and increase meat production using the same amount of feed resources.

MATERIALS AND METHODS

To determine the cellular localization of *DNTTIP1*, immunohistological staining of C2C12 cells transfected with *DNTTIP1* was performed. Electroporation as a method of transfection increases the permeability across the cell membrane so the plasmids are more readily taken up. The plasmid encoded *DNTTIP1* on the amino terminal end and a V5 epitope tag on the carboxyl terminal end. Electroporations were done using the Neon Transfection System (Life Technologies). The plasmid concentration was greater than 2 µg/µL so the plasmid didn't surpass 10% of the transfection volume. Cells were resuspended in Neon buffer R along with the *DNTTIP1* plasmids. Next, the resuspended cells and plasmids were drawn into the Neon pipette and placed in the electroporation device. Upon completion of electroporation, the transfected myoblasts were resuspended in growth media containing 10% fetal calf serum. Finally, the cells were fixed in 4% formaldehyde in phosphate buffered saline (PBS), permeabilized with 1% triton X 100 in PBS for 5 minutes, and blocked for 1 hour with 3% bovine serum albumin in PBS.

Expression of the *DNTTIP1* protein was detected using a rabbit polyclonal primary antibody against *DNTTIP1* or a mouse monoclonal antibody against V5 epitope protein sequence. The primary antibodies were detected using the following secondary antibodies: a chicken anti-rabbit antibody conjugated to rhodamine for *DNTTIP1* and a goat anti-mouse antibody conjugated to FITC for the V5 epitope tag. The cells were treated with DAPI (4',6-diamidino-2-phenylindole), which binds DNA to stain the nuclei of the cell. The V5 epitope tag was codetected along with the *DNTTIP1* protein using a fluorescent microscope for confirmation of cellular localization, as it was fused to the carboxyl end of the plasmid construct.

The effect of increased expression for *DLK1* and *DNTTIP1* on myosin promoter activity was tested in primary mouse myotubes. Two myosin promoter-luciferase reporter plasmids containing either 3.5 kb of the rat *Myh7* (Type I) promoter (Swoap, 1998) or 2.5 kb of the mouse *Myh4* (Type IIb) promoter (Hasegawa, Lee, Jobe, Markam, & Kitsis, 1997) were co-transfected with a Renilla luciferase plasmid and one of the two effector plasmids (*DLK1* or *DNTTIP1*). The amount of effector plasmid was titrated, and a null vector GW-CAT was used to keep the amount of transfected DNA constant. After electroporation, the myoblasts were replated in media with 10% fetal calf serum overnight and then differentiated into myotubes by the addition of media containing 2% horse serum for 48 hours. To measure luciferase activity following differentiation, two reagents were needed: Dual-Glo buffer and Stop & Glo. Both reagents were made by dilution in a 1:1 ratio with PBS. The 96 well plate containing the myotubes was washed once with PBS before the Dual-Glo buffer was added. The Tecan microplate reader was used to measure chemiluminescence levels. The myosin luciferase activity was quantified first, and then the Stop & Glo buffer was added to measure the renilla luminescence. Luciferase values were adjusted for transfection efficiency using the Renilla luminescence, and treatment differences were determined using Dunnett's t-test for multiple comparisons.

RESULTS AND DISCUSSION

The first objective, cellular localization of *DNTTIP1,* determined that *DNTTIP1* is in fact localized to the nucleus. Transfection of the *DNTTIP1*-V5 plasmid produced readily detectable protein expression in C2C12 muscle cells (see Figure 3). Detection of the C-terminal V5-epitope (green) tag in the *DNTTIP1*-V5 fusion protein indicates expression of the full length *DNTTIP1*-V5 protein. Detection of *DNTTIP1* using the anti-*DNTTIP1* antibody (red) shows that the plasmid expresses the *DNTTIP1* protein. DAPI binds DNA and stains the nucleus of all cells. All three photos (Figure 3, B, C, and D) were merged to show the overlap of fluorescent signals for *DNTTIP1* expression and nuclear location (Figure 3A). All fluorescent signals overlapped as seen in Figure 3; therefore, *DNTTIP1* is determined to be located in the nucleus. Since *DNTTIP1* was determined to be a nuclear factor, luciferase assays were used to find out if it has an effect on myosin gene expression.

The hypertrophied muscles of callipyge sheep lambs have elevated levels of *DLK1* and *DNTTIP1,* along with a number of other genes including *MYH4*. Plasmids expressing *DLK1* and *DNTTIP1*-V5 were co-transfected with myosin promoter constructs to determine if they could alter myosin promoter activity. Measurement of luciferase activity acts as a reporter for the promoter activity using a dose titration for the two effector genes *DLK1* or *DNTTIP1*-V5 relative to a null control gene (GW-CAT). *DLK1* significantly decreased *Myh7*-luciferase expression as the amount of transfected *DLK1* increased (Figure 4). The largest amount of *DLK1* plasmid (48% of total transfected DNA) induced a significant decrease in *Myh7* promoter activity. There was no difference between the control and 24% *DLK1* treatment and a trend for a difference between the 24% and 48% treatments. *DLK1* expression significantly increased *Myh4* promoter activity as the quantity of *DLK1* increased (Figure 4). The 48% treatment was significantly different from 24% treatment and the control. The 24% treatment was not significantly different from the control. These results are consistent with endogenous gene expression in callipyge sheep. *Myh4* promoter activity is increased in callipyge sheep, whereas *Myh7* promoter activity is decreased.

Overexpression of the *DNTTIP1*-V5 significantly lowered *Myh7*-luciferase expression relative to the control at both the 12% and 24% treatment levels (see Figure 5). There was no significant difference between the 12% and 24% quantities in *Myh7*-luciferase expression. *DNTTIP1* significantly increased *Myh4*-luciferase expression relative to the control at both treatment levels. The effect of *DNTTIP1* expression, causing decreasing *Myh7* expression and increasing *Myh4* expression, was consistent with myosin gene expression in callipyge sheep muscle.

DNTTIP1 has been shown to play a role in the regulation of callipyge sheep muscle development. Immunohistological staining of transfected cells confirmed that *DNTTIP1* localizes to the nucleus, which is consistent with its role as a nuclear transcriptional cofactor. The overall higher level of *Myh7* activity relative to *Myh4* activity was consistent with endogenous gene expression observed in cultured mouse primary

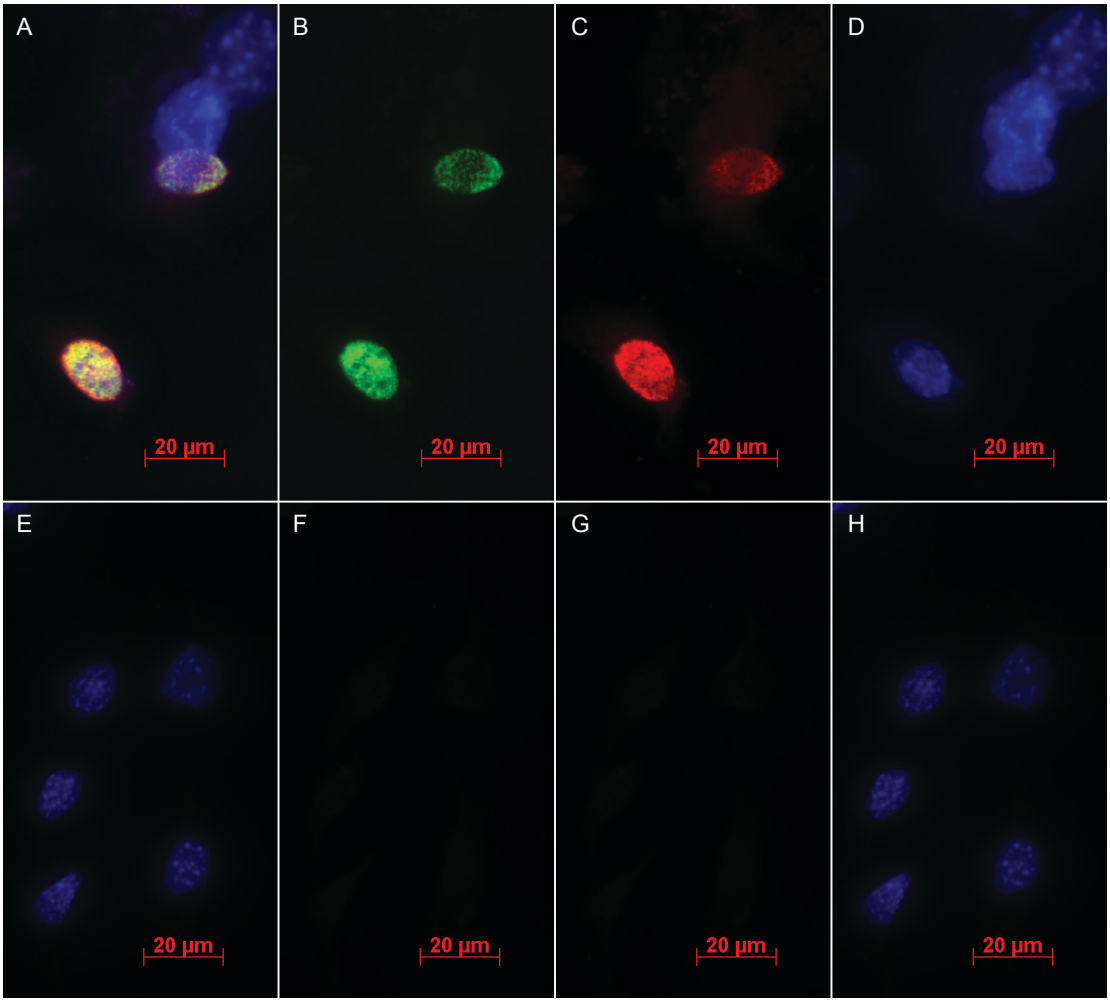

Figure 3. Nuclear localization of *DNTTIP1* in C2C12 muscle cells. Cells transfected with a plasmid expressing a *DNTTIP1*-V5 fusion protein show nuclear localization of the protein. A) merged imaged from B–D; B) detection of the V5 epitope tag; C) detection of *DNTTIP1*; D) DAPI staining of nuclei; E) merged image F–G; F) V5 secondary antibody only; G) *DNTTIP1* secondary antibody only; H) DAPI staining of nuclei.

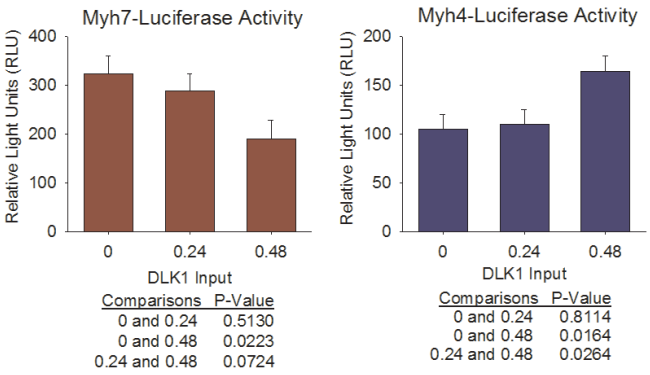

Figure 4. Effect of DLK1 expression on myosin promoter activity in mouse primary myotubes. Comparisons of each treatment are shown under each graph. *DLK1* significantly decreased *Myh*7-luciferase activity at the higher concentration (48%) relative to the control. In the *Myh4*- luciferase assay, *DLK1* significantly increased *Myh4* promoter activity at the 48% concentration relative to the control.

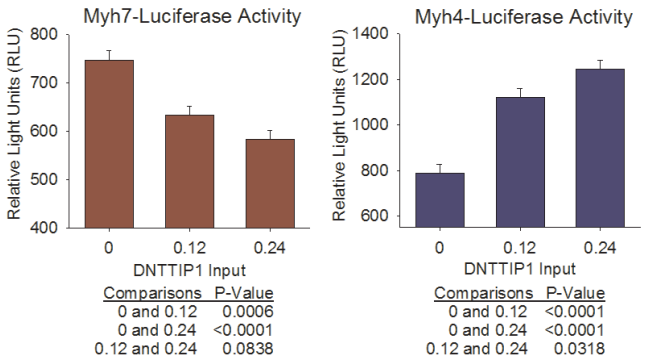

Figure 5. Effect of *DNTTIP1* on myosin promoter activity in mouse primary myotubes. The comparisons for each treatment are shown under each graph. *DNTTIP1* significantly lowered *Myh*7-luciferase activity relative to the control at both the 12% and 24% quantities, but there was no significant difference between the 12% and 24%. *DNTTIP1* significantly increased *Myh4*-luciferase activity at both concentrations relative to the control.

myotubes and supports the suitability of the cell culture model to test effector genes for their influence on myosin promoter activity. The expression of *DLK1* and *DNTTIP1* increased the activity of the *Myh4* promoter (fast twitch glycolytic) and decreased the activity of the *Myh7* promoter (slow twitch oxidative) in cultured mouse primary myotubes, which supports the involvement of *DLK1* and *DNTTIP1* in controlling the callipyge phenotype. Callipyge sheep have increased *Myh4* (fast twitch glycolytic fibers) and decreased *Myh7* (slow twitch oxidative fibers).

The effect of *DLK1* and *DNTTIP1* was consistent with the changes in myosin gene expression patterns found in callipyge sheep muscle. These results support the hypothesis that *DNTTIP1* can directly regulate and influence the expression of myosin genes. This regulation may involve an interaction of the *DNTTIP1* transcriptional cofactor with AT-rich sequences in the myosin gene promoters, as *DNTTIP1* binds with high affinity to AT-rich sequences. Knowing that *DNTTIP1* is involved in the characteristic callipyge muscle hypertrophy is part of piecing together the muscle growth regulatory pathway. In characterizing the muscle growth pathway, scientists can target specific parts to achieve desired goals. This could be of use to producers looking to increase feed efficiency in their lambs. Further work will be directed at analyzing other potential genes thought to play a role in muscle hypertrophy using this effector gene/myosin-reporter model system.

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